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The Combined Effects of Leptin and Coenzyme Q10 in Ameliorating Obesity- Induced Infertility in Female Rats

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The Combined Effects of Leptin and Coenzyme Q_{10} in Ameliorating Obesity- Induced Infertility in Female Rats

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by

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ABSTRACT

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Infertility is one of the major problems of obesity. Studies have shown that administration of leptin reversed obesity-induced infertility in rats and mice. Coenzyme Q_{10} (CoQ_{10}) is an antioxidant and also supplies the energy needed for ovulation and embryo development. We hypothesized that leptin when combined with CoQ_{10} could greatly improve obesity-induced infertility. The results showed a significant decrease in food intake, body weight, and the regular estrous cycle was restored after treatment with leptin+CoQ_{10}. There was a significant increase ($p<0.05$) in follicle stimulating hormones after treatment. It was observed that CoQ_{10} significantly ($p<0.05$) down-regulates the expression of β-catenin in the ovary while E-cadherin failed to be expressed. In conclusion, administration of leptin with CoQ_{10} can improve fertility in obese infertile female rats. This study could provide a novel therapeutic strategy for the treatment of infertility and formulation of new drugs for the treatment of obesity-induced infertility in females.

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DEDICATION

This work is dedicated to God Almighty and to everyone that has contributed to knowledge.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Leptin

Leptin is a 16kDa protein product produced by obese (*ob*) *gene*. It was first identified and characterized in 1994 by Zhang *et al* [82]. Leptin is secreted primarily by adipose tissue (fat cells) mainly white adipose tissue [82] and produced in a small amount by the skeletal muscle, mammary gland, placenta and stomach [13]. It plays a significant role in the physiologic functions of the body by regulating food intake, body weight and energy expenditure [17]. Leptin regulates the neuroendocrine, metabolic, reproductive as well as immunological processes [13]. Leptin exerts its main effects on the nuclei of the basomedial hypothalamic area of the brain where leptin receptors are located [19].

The Role of Leptin in Food Intake and Energy Expenditure

The major function of the leptin is to maintain homeostatic control of fats by regulating the food intake, body weight and energy expenditure [17]. Leptin is secreted primarily by the adipose tissue (fat cells), released into the blood stream and then transported to the hypothalamus of the brain[2]. One of the major functions of the hypothalamus is to regulate food intake and energy expenditure by recognition of peripheral signals such as leptin [43]. Once leptin is released into the blood stream by the adipose tissue, it is transported to the brain where it binds with the neurons of the leptin receptors (LEPR) located within the nuclei of the basomedial hypothalamus; arcuate (ARC), dorsomedial hypothalamic and ventromedial hypothalamic nuclei of the brain. The neurons relay information about the energy status of the body to the brain [19.] The neurons exhibit two forms; orexigenic (increases appetite) and anorexigenic (decreases

appetite) neuropeptides. orexigenic neuropeptides expression modulated by leptin include Neuropeptide Y (NPY), agouti-related protein (AGRP), melanin-concentrating hormone (MCH), orexin, galanin and galanin-like peptide (GALP) [19]. Anorexigenic peptides expression modulated by leptin include pro-opiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript, neurotensin, corticotropin-releasing hormone (CRH) and brain-derived neurotrophic factor [7]. Studies have shown that central administration of orexigenic neuropeptides such as NPY increase food intake and decrease the energy expenditure[53]. Central administration of leptin causes leptin to bind with the LEPRs located within the nuclei of the basomedial hypothalamus (particularly ARC), thereby inhibiting secretion of orexigenic neuropeptides (which stimulate appetite) but stimulate the release of anorexigenic neuropeptides to decrease appetite. This results in decrease in food intake and increase in energy expenditure with respect to the total body fats and body mass index [53]. NPY is associated with the regulation of gonadotropin-releasing hormone (GnRH) by binding directly with GnRH via the brain and / or pituitary and thus regulates gonadotropin secretion (Fig. 2). The GnRH stimulates anterior pituitary to release follicle stimulating hormone (FSH) for folliculization and luteinizing hormone (LH) stimulates the gonads in preparedness for ovulation (Fig. 2). In 1996, Schwartz et al. demonstrated that the level of circulating leptin in peripheral blood is dependent on total body fat and body mass; The higher the total body fat and the body mass index the higher the circulating leptin [64]. When fat mass falls, the circulating leptin level falls, stimulating appetite and suppressing energy expenditure until the fat mass is restored. However, when fat mass increases, circulating leptin level also increases, suppressing appetite until the weight is lost. This

mechanism by leptin helps the body to maintain and regulate homeostatic control and energy balance of the body [47]. Other factors that causes an increase in circulating leptin include food intake [41,40], glucose uptake [73] and gender- higher in females than males [56]. However, exercises[37] and an increase in age[56] can also decrease the level of circulating leptin.

Leptin Receptors

Leptin receptors (LEPRs) are classified as class 1 cytokine receptors because it has a common downstream signal pathway and homologous structure with interleukin 6 [36]. LEPRs are located mainly in the hypothalamus, site for control of appetite, reproduction, growth and metabolism [43]. LEPR are also expressed in small amounts in other parts of the brain such as the cerebellum, anterior pituitary and other parts of tissues such as placenta and stomach [43]. Leptin receptor family arises from alternative splicing, consists of six isoforms called obese receptors (*ob-R*) isoforms and are grouped into two forms; short and long forms. The short forms include *ob-Ra, ob-Rc, ob-Rd, ob-Rf*, and soluble form, *ob-Re* which circulates in the plasma. The only long isoform, *ob-Rb* is mainly expressed in the hypothalamus [17]. The *ob-Rb* contains a long intracellular domain which has two protein motifs that signal via Janus-activated kinases (JAK)-signal transducers and activators of transcription (JAK-STAT) pathway [70]. The short intracellular isoforms signal via phosphatidyl inositol-3 (PI-3) kinase or mitogen-activated protein kinase (MAPK) pathway. The short forms do not activate JAK-START pathway because they contain only one of the two JAK – binding domains [70].

Leptin binds with LEPR majorly in the hypothalamus and activates different signals that relay information regarding the physiological status of the body to the brain [2].

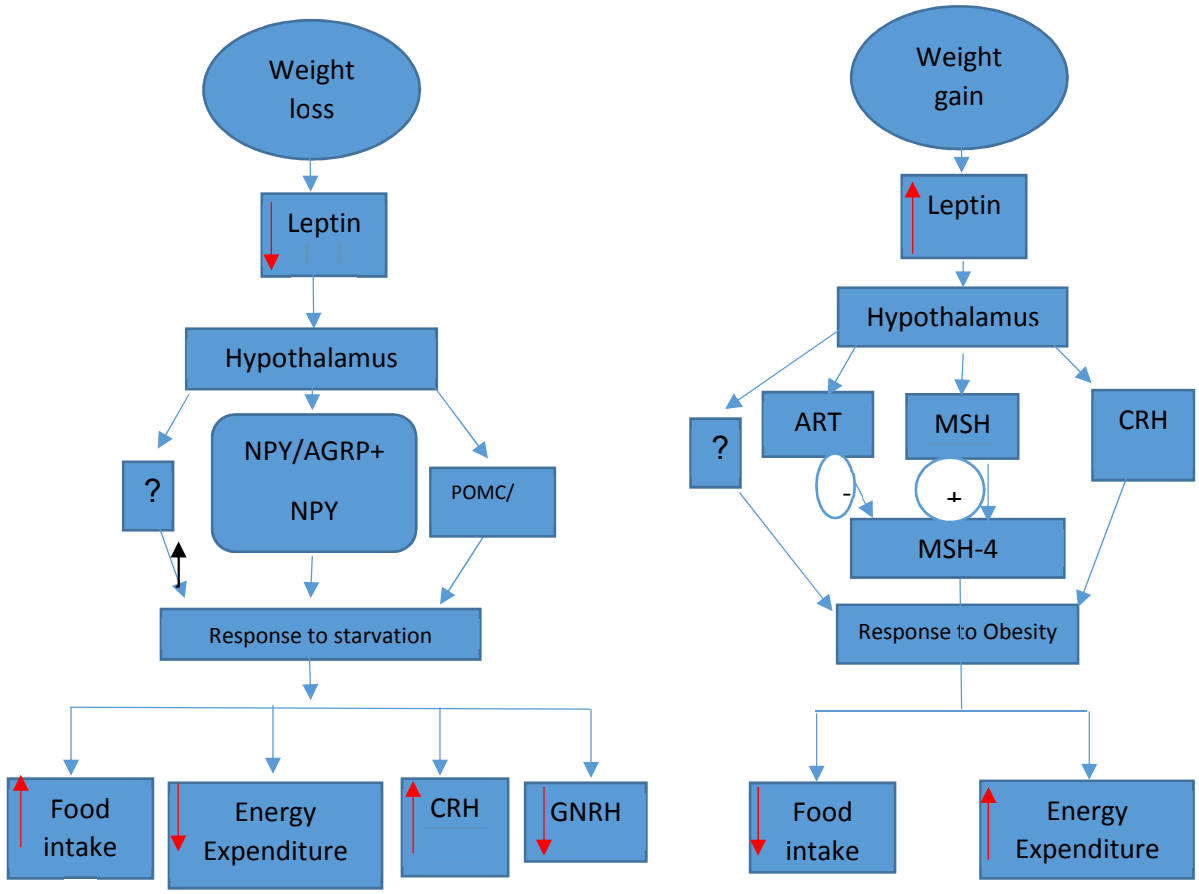

Fig. I. Biological response of leptin in low (starvation/fasting) and high levels (obesity).

During starvation/fasting/hunger, there is a decrease in adiposity which leads to a decrease in concentration of leptin in the blood. Leptin binds to leptin receptors in the hypothalamus of the brain and conversely activates several pathways; increase NPY/AgRP, neuropeptide Y/Agouti-related peptide; decrease POMC, proopiomelanocortin; CART, cocaine- and amphetamine-regulated transcript. This pathway relays the information about the energy status of the body to the brain and

ultimately leads to increase food intake and a decrease in energy expenditure as well as metabolism. However, in conditions such as obesity, the reverse is the case. Several hormones are involved in leptin response to energy status. These include; gonadotropin releasing hormone (GnRH), corticotrophin release hormone (CRH), melanin-concentrating hormone (MCH), thyroid-stimulating hormone (TSH), thyroid-releasing hormone (TRH), adrenocorticotropic hormone (ACTH), growth hormone (GH); IGF-I, insulin-like growth factor I as well as reproductive hormones such as luteinizing hormone (LH), follicle-stimulating hormone (FSH) and estrogen [32, 64].

Role of Leptin in Obesity, Infertility and Reproduction

One of the neglected underlying causes of infertility is obesity. Infertility is associated with obesity and overweight, especially when it occurs at the early stage of life or during puberty [57]. Obesity reduces the chances of ovulation and pregnancy in women that ovulate regularly [26]. Previous studies suggested that the impairments in fertility caused by obesity has its reproductive targets on hypothalamus, ovary, ovarian follicle, oocyte, embryo as well as uterine endothelium [79,8, 77, 61, 71]. The role of leptin in infertility was first demonstrated in 1997 by Chehab [18]. It was observed that defective *ob/ob* female mice whose failure to make leptin were attributed to a gene mutation that prevented the mice from ovulating and thus resulted to infertility due to hormonal abnormalities (gonadotropin secretion impairment) [18]. Fertility was restored after administration of exogenous leptin[18]. The success of reproduction is dependent on food intake and energy reserves of the body [57]. Pasquali and Gambineri study showed that excessive fat accumulation in adipose tissue (fat cells) and the associated release of high concentrations of free fatty acids into various organs can impair fertility [57].

Evidently, there is a linkage between nutritional status, obesity, and reproduction [55]. Previous researchers have also demonstrated that leptin is involved in reproduction; neuroendocrine regulation of prolactin, luteinizing hormone, estrogen, follicle stimulating hormone, progesterone and GnRH [1,24]. Leptin has its effects on the hypothalamus–pituitary–gonadal (HPG) axis, viz-a-vis, gonadal, adrenal, thyroid, pancreatic islet, and growth hormone [17]. Study by Yu *et al.* in rats showed that leptin can directly stimulate the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus and luteinizing hormone (LH) from the pituitary [80]. This finding was also corroborated in a study by Zieba, Amstalden, & Williams in cattle [83]. The hypothalamic control of gonadotropin secretion is regulated by GnRH [80] which has effects on reproduction[17].

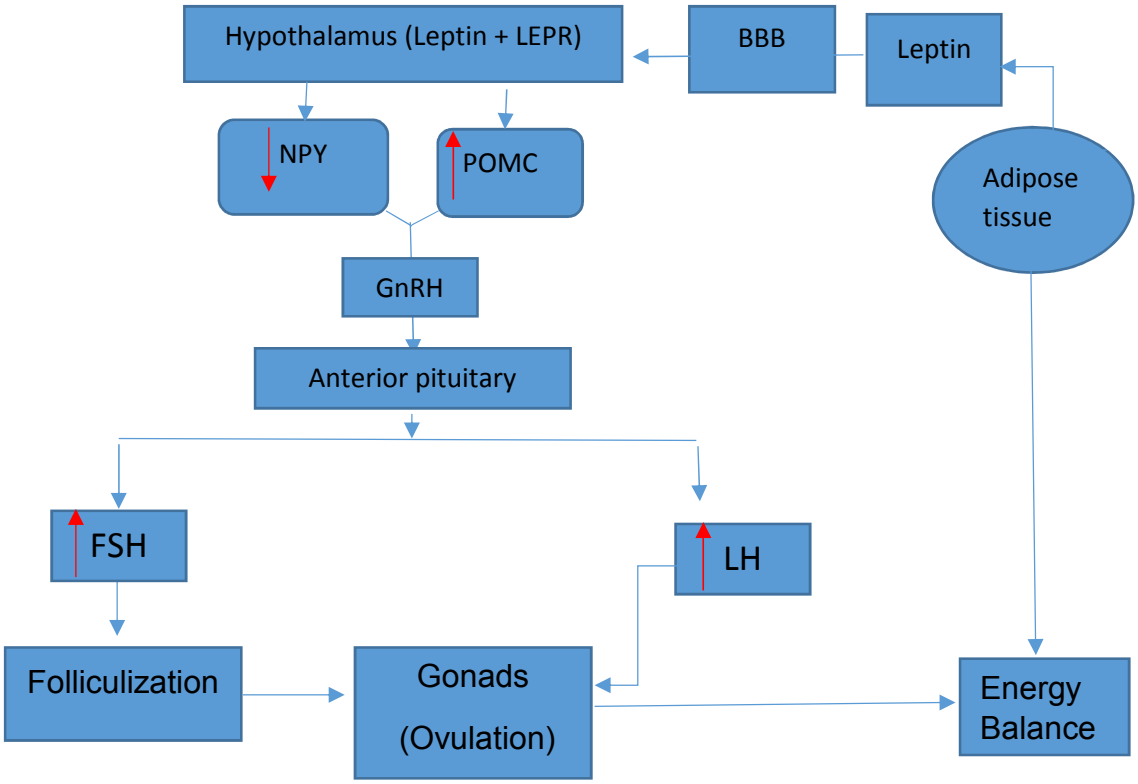

Fig. 2. Response of leptin in the reproductive system.

The adipose tissue stimulates the synthesis of leptin which is then transported to the brain (hypothalamus) via the blood-brain barrier (BBB) where different neuropeptide signals are being activated. Leptin binds with leptin receptors in the preoptic area of the hypothalamus and causes an increase in gonadotropin-releasing hormone (GnRH) secretion. The GnRH stimulates anterior pituitary to release follicle stimulating hormone (FSH) for folliculization and luteinizing hormone (LH) for stimulation of the gonads in preparedness for ovulation (Fig. 2).

Epithelium Cadherin and Beta Catenin

Epithelium Cadherin (E-cadherin)

Cadherin belongs to the superfamily of transmembrane glycoproteins that contain repeated units of approximately 100 residues in their extracellular domain. The primary function of cadherin is to mediate calcium dependent cell-cell interaction and also play key roles in normal tissue development and proliferation of many organs [68]. Disruption of cadherin function has been linked to different kind of developmental and pathological conditions such as cancer [76]. The classical cadherin subfamily includes E, P, R, B and N and other different subtypes. The subtypes are given the name depending on the site they are found. For example, E- cadherin, the most studied member of the family is found in the epithelial tissue [45]. N-cadherin is found in the neuron; P-Cadherin is found in the placental; R-cadherin in the retina; VE- Cadherin in vascular endothelial [4]. E-cadherin also known as cadherin 1 is a protein that is encoded by CDH1, tumor suppressor gene in human [76]. It consists of five repeated cadherin residue, EC1 to EC5 in the extracellular domain, one transmembrane domain, and an

intracellular domain (cytoplasmic tail) that interact with p120-catenin and beta catenin. The intracellular domain contains a highly-phosphorylated region and facilitates the binding to β-catenin, and thus promotes cell to cell interaction with E-cadherin function. E-cadherin down-regulation decreases the strength of cellular adhesion within a tissue, resulting in an increase in cellular motility [76].

Beta Catenin

Beta (β-) catenin also known as catenin beta-1 is a CTNNB1 protein encoded by CTNB1 gene in human [46]. It was discovered that mammalian β- catenin is homologous both in structure and function with drosophila protein called *armadillo*. *Armadillo* (ARM) is a repeated unit of amino acids (approximately 40) folded into single, elongated, rigid protein domain. The *armadillo* is involved in mediating the morphogenic effects of *Wingless/Wnt* [38]. β-catenin is a subtype of catenin protein family and homologous to γ-catenin (plakoglobin). It is expressed in tissue such as cardiac muscle and ovary [59]. The β-catenin is a key downstream effector protein in the *Wnt* signaling pathway that has been linked to different kind of developmental and pathological conditions such as cancer [16]. β-catenin is involved in two major biological processes: early embryonic development [75] and tumorigenesis [60]. β-catenin is involved in cell to cell adhesion as well as gene transcription [74].

E-cadherin/β-Catenin Complex

E-cadherin cytoplasmic domain is linked to the actin cytoskeleton through the β catenin*, Y* catenin and p120 catenin to form cell to cell adhesion junction. The β and *Y*-catenin is linked to α- catenin to form E-cadherin/β-catenin complex which in turn linked

to the actin cytoskeleton. This complex formation enables the E-cadherin to maintain the structural integrity and polarity of the epithelial cells and tissues. β and *Y*-catenin play structural roles in the junctional complex while p120 regulates the adhesive activity and trafficking [74].

E-cadherin/β-Catenin Complex Expression in Rat Ovary

Expression of E-cadherin/β-catenin complex is involved in the development of tissues and proliferation of organs [68]. E-cadherin/β-catenin complex expression is increased in the theca and interstitial cells of the rat ovaries during follicular development. However, granulosa cell has more expression of β-catenin [67]. The cytoplasmic tail of E-cadherin (plasma membrane) is linked to β-catenin (nucleus) to form a complex that helps in the regulation of cytoskeletal structures and intracellular signaling. Studies have shown that modulation of E-cadherin/β-catenin complex expression forms an integral component of remodeling processes which include folliculogenesis and corpus luteum formation in the ovary [67]. During these processes, E-cadherin/β-catenin complex is under the influence of hormones such as estrogen[30].

Coenzyme Q_{10}

Coenzyme Q_{10} (CoQ_{10}) is a fat-soluble vitamin-like compound [3] produced naturally in most cells of the body with higher concentration found in tissues with high energy stores (mitochondria) such as the brain, heart, liver, kidney [27]. The oocyst (ovary) has the highest number of mitochondria and mitochondrial DNA more than any cells with high energy requirements such as muscle cells and neurons [10]. CoQ_{10} can also be found in small amounts in diets such as meats (especially organ meats),

sesame oil, soybeans, nuts, dark leafy green vegetables like spinach, kale and broccoli, seafood, and avocado [14]. CoQ_{10} is made up of benzoquinone and a hydrophobic tail that comprises of isoprenoids units which can be reduced to ubisemiquinone and finally reduced to ubiquinol which is the biological active [69]. There are two forms of CoQ_{10}; ubiquinone and ubiquinol. CoQ_{10} starts off as ubiquinone and converted to the more potent active antioxidant, ubiquinol in the cell. Most CoQ_{10} supplements are available in the form of ubiquinone. ubiquinol does not need to be converted by the body. The main function of CoQ_{10} is the transfer of electron in the mitochondrial electron transport chain, used for adenosine triphosphate (ATP) synthesis through ATP synthetase. The ATP synthesis is responsible for creating cellular energy. CoQ_{10} is also a powerful lipid-soluble antioxidant, its tissue concentration is about five to ten folds higher than the main fat- soluble antioxidants such as vitamin E [9]. It inhibits lipid peroxidation by providing protection and scavenging free radical or oxidant damage caused by reactive oxygen species (ROS) or reactive nitrogen species (RNS) in the mitochondria [42]

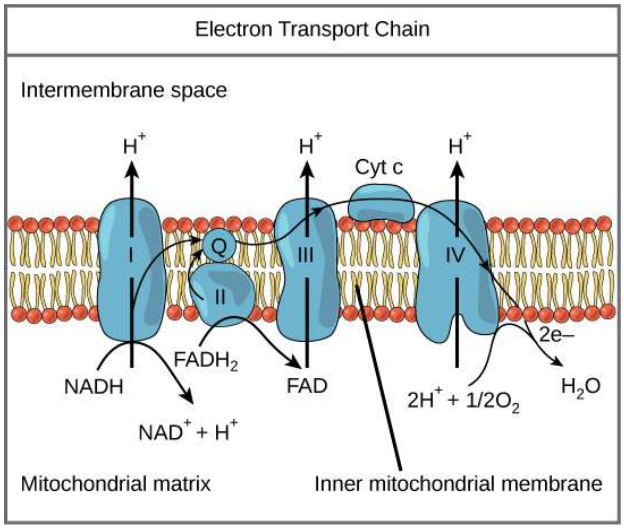

Fig. 3. The role of CoQ_{10} in mitochondria Electron transport chain. Molnar et al. (2013) [85]

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CoQ_{10} has shown to be a promising compound in the management and treatment of a wide range of diseases partly or directly caused by mitochondrial disorder such as ischemic heart disease, muscular degeneration, Parkinson, Alzheimer, epilepsy, Diabetes, congestive heart failure, hypertension, cancer, as well as infertility [66,10,14]. Increase in age, as well as administration of drugs such as Statins decrease the concentration of CoQ_{10} in tissues [49]. CoQ_{10} increases ATP synthesis in the mitochondria thus enhancing mitochondrial function and decreases the oxidative stress that causes DNA damage in both animals and human [10]. Studies have shown that the presence of ubiquinol in the cell membrane help to reduce cell and DNA damage caused by free radicals, which has a negative impact on health [10]. Bentov, Esfandiari, Burstein, & Casper (2010) study showed that mitochondrial DNA damage can be reversed by the supplementation with antioxidants particularly CoQ_{10} [10]. In reproduction, CoQ_{10} increases the availability of energy for egg quality, sperm motility, maturation of oocyte, embryo development as well as implantation in infertile females[10]. The survival and quality of oocyte in the ovary rely extensively on the production of ATP, therefore, an increase in the concentration of CoQ_{10} in the plasma could produce a desirable and positive outcome in fertility. The study conducted by Burstein et al., (2009) in mice concluded that supplementation of CoQ_{10} improves mitochondrial function as well as an ovarian response in old mice. It has also been shown that combination of CoQ_{10} with other supplements such as Zinc, lipoic acids, omega-3 fatty acid, vitamins C and E improves mitochondrial disorders than when used alone [1,68]. Also, findings from

Rodriguez *et al*. study in 2007 showed that combination of Co_{Q10} with other supplements such as lipoic acid and creatine was more effective in the treatment of mitochondrial disorders than the lone pair in health related diseases [62]. Abdali, Samson, and Grover (2015) study also described that antioxidants supplementations are beneficial in the management of type 2 diabetes and obesity, especially when supplemented with a combination of antioxidants such as Vit C, E, Lipoic acid, Zinc than CoQ_{10} alone. The study also recommended testing this hypothesis in high energy health related disease such as infertility [1]. Tarnopolsky (2008) also corroborated other researches that supplementation of CoQ_{10} with another compound could greatly improve and produce positive impacts in fertility than lone administration [69]. It is important to conduct this research among females because females are the first target during diagnosis of infertility among couples. With significant evidence showing a positive effect of leptin in reversing obesity-induced infertility and CoQ_{10} showing a promising outcome in the treatment of some disease conditions such as male infertility, it is important to test if the combination of leptin with CoQ_{10} could actually yield a more positive outcome in obesity-induced infertility in female rats. This study is novel research as there is no available data on effects of the combination of leptin and CoQ_{10} in obesity-induced female infertility. This study could provide a therapeutic strategy for the treatment of infertility as well as formulation of new drugs for the treatment of obesity-induced infertility in females.

Hypothesis

The combination of leptin and CoQ_{10} greatly improve obesity-induced infertility in female rats.

Objectives

To measure the spleen weight, body weight and food intake.

To measure the total antioxidants level in the blood.

Evaluation of expression of β- catenin and E-cadherin proteins in the ovary.

Evaluation of expression of leptin receptors in the hypothalamus.

Determination of plasma level of luteinizing, estrogen and follicle stimulating hormones.

Evaluation of total body composition of each group of the rats.

CHAPTER 2

MATERIALS AND METHODS

Animals Model

Twenty-one female Sprague-Dawley rats of weight range 150-250g were acquired from Harland and used in this study (Protocol #100501). The animal use protocol was approved by the University Committee on Animal Care (UCAC) of East Tennessee State University. Animals were housed individually and kept in a well-controlled, specific pathogen-free room, located in the Brown Hall DLAR facility at ETSU. Food and water were made available ad libitum. Four of the rats were fed regular chow diet (RCD), and the remaining seventeen were fed with high-fat diet (HFD) to induce infertility.

Determination of Oestrus Cycle

A normal regular oestrus cycle of rats is between 4-5 days and consists of proestrus, estrus, metestrus, and diestrus stages (Fig. 4). Vaginal cytology is used to determine different stages of the estrous cycle. A vaginal cytology was conducted using a wet mount process to visualize the vaginal cells. A small amount (10µl) of saline solution was introduced into the vaginal orifice of each rat and then aspirated and placed on a clean dry grease free slide. The cells were then checked using a compound microscope at 10X and 40X for analysis.

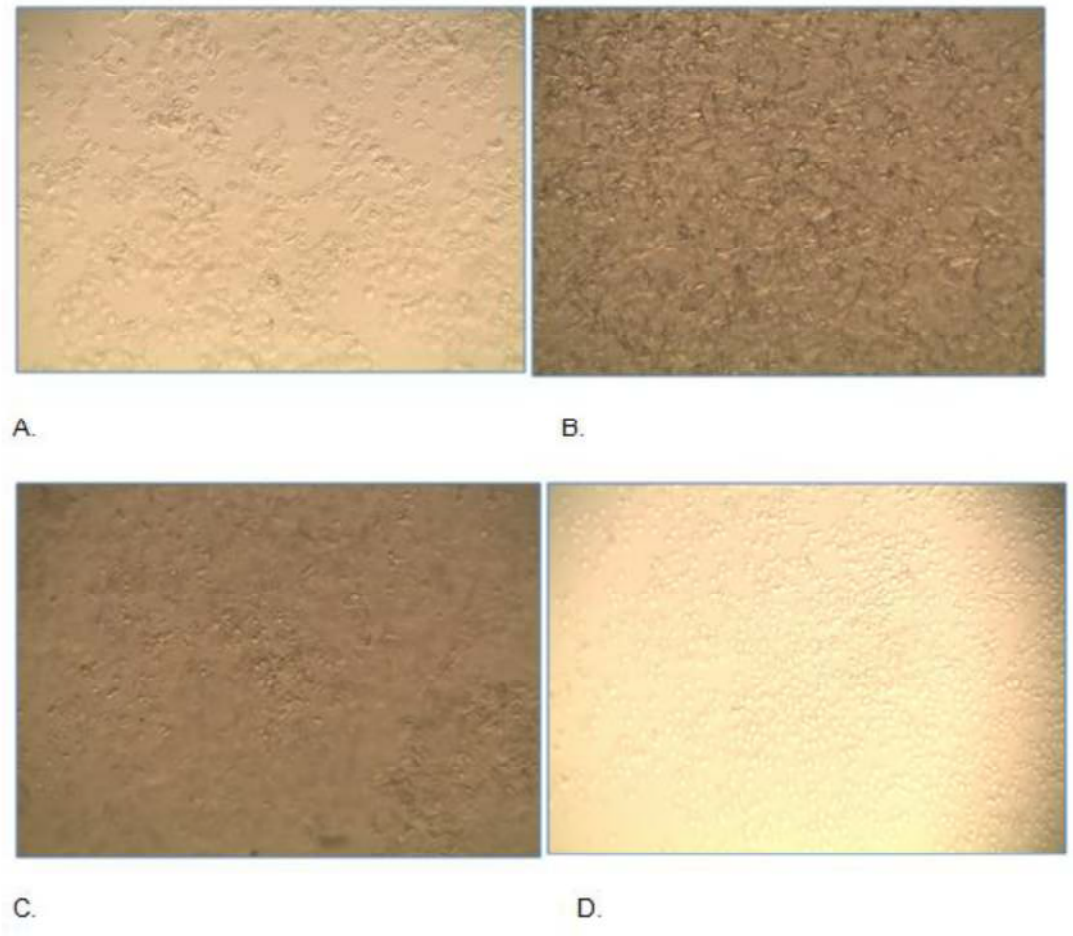

Fig 4. Microscopy view of oestrus cycle stages

Vaginal smears pictures that were taken at 10X using digital camera microscope.

Each of the pictures represents stages of the oestrus cycle.

(A). Proestrus: Predominance of nucleated epithelial cells.

(B). Estrus: Predominance of anucleated cornified cells.

(C). Metestrus: Nucleated epithelial cells, anucleated cornified cells, and leukocytes.

(D). Diestrus: Predominance of leukocytes.

Irregular Oestrus Cycle

The irregularity of the oestrus cycle of the rats fed with HFD was first noticed toward the end of the 12th week of the study (Fig. 5), which signified the onset of infertility. Infertility occurs when any of the stages; proestrus, estrus, metestrus, or diestrus of the 4-day oestrus cycle is skipped particularly estrus phase or maintained for more than two days. The ability of each rat to go through a 4-day cycle without skipping any stage signifies a regular oestrus cycle. The rats' body weight and food intake were measured twice weekly while the estrus cycle checked daily until the oestrus cycle became disrupted.

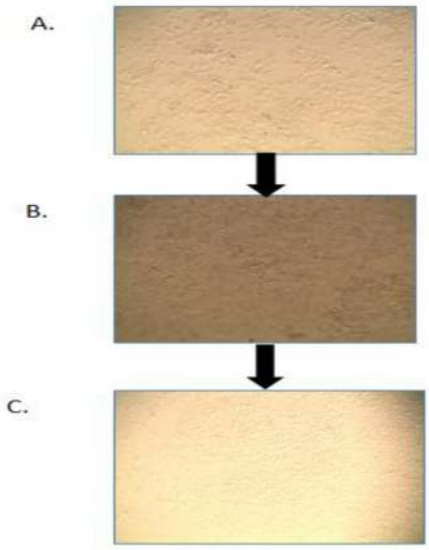

Fig 5. Representation of an irregular oestrus cycle.

The irregular oestrus cycle moved from (A) Proestrus: predominance of nucleated epithelial (skipping estrus stage) to (B) Metestrus: nucleated epithelial cells, anucleated cornified cells, and few leukocytes, and then (C) Diestrus: predominance of leukocytes.

Animals were divided into five groups and treated as follows:

Group I: Fed regular chow diet (RCD), received d-alpha tocopheryl polyethylene glycol 1000 succinate (TPGS) (10mg/ml/kg/b.w/day) intraperitoneally (i.p).

Group II: Fed high fat diet (HFD), received TPGS (10mg/ml/kg/b.w/day) for two weeks intraperitoneally (i.p)

Group III: Fed HFD, received leptin (100ug/ml/kg/b.w/twice/day) for two days i.p.

Group IV: Fed HFD, received CoQ_{10} (100mg/ml/kg/b.w/day) for two weeks i.p

Group V: Fed HFD, received leptin (100ug/ml/kg/b.w/twice/day) for two days and CoQ_{10} (100mg/ml/kg/b.w/day) i.p.

Table 1: Groups and treatments administered

Group	Name	Diet	Leptin	CoenzymeQ10	TPGS
I	RCD	CHOW	-	-	+
II	HFD	HFD	-	-	+
III	HFDleptin	HFD	+	-	+
IV	HFDCoQ10	HFD	-	+	+
V	HFDleptin+CoQ10	HFD	+	+	+

High-fat diet (HFD), regular chow diet (RCD), Tocopheryl polyethylene glycol 1000 succinate (TPGS). TPGS (10mg/ml/kg/b.w/day), leptin (100ug/ml/kg/b.w/twice/day) CoQ_{10} (100mg/ml/kg/b.w/day).

Group I was fed a regular chow diet (RCD) while groups II to V were fed a high-fat diet (HFD) for 14 weeks to induce infertility. Estrous cycle was checked daily. Food intake and body weight measured twice weekly before and after treatments. All the animals were sacrificed fourteen days after the last day of treatments (18 weeks). Blood, tissues and carcasses were collected for further analysis.

Blood and Tissue Collection

Blood samples were collected from each rat into a 5 ml EDTA tube and placed on ice. It was later centrifuged at room temperature for 10 minutes at 2000Xg using Sorvall Legend XTR centrifuge (Thermo scientific, USA). Once centrifuged, the supernatant (plasma) was pipetted into 3ml glass amber vials and stored at -30 °C for further analysis of leptin, antioxidant status, ferric reducing ability of plasma (FRAP) and reproductive hormones concentrations. The red blood cell (RBC) pellet at the bottom of the EDTA tube was diluted with equal parts of normal saline (0.9% NaCl), vortexed briefly to disrupt the RBC pellet, and centrifuged for 5 minutes at 2000Xg. The clear supernatant was removed and discarded. This process was repeated two more times with the clear supernatant removed and discarded each time. Red blood cell pellet of 100 µl was transferred to clear 150mm screw-top test tube with Teflon lined caps and 2ml of n-hexane, 95% (Fisher Scientific, cat. # 130939) was. This process was done in duplicate and stored at 30^{O}C prior to fatty acid methylation of the RBC.

FRAP Assay

The plasma was evaluated for antioxidant capacity using a spectrophotometric technique, ferric reducing ability of plasma (FRAP) assay, as described and modified by Benzie & Strain (1996). It works on the principle that the intensity of the color produced is directly proportional to the concentration of antioxidant present in the sample read at an absorbance of 593nm. FRAP assay is used to determine total antioxidant capacity in serum/plasma samples. The FRAP reagent was prepared fresh before each series of measurements to ensure validity. The reagent contained the following materials: 200 ml of acetate buffer (300mM, pH 3.6), 20 ml TPTZ solution (0.062 g 2,4,6-tripyridyl-s-

triazine, or TPTZ, in 20ml 40mM HCl), 20 ml FeCl3 solution (0.2748 g FeCl3-6H2O in 50 ml DDW), and 24ml DDW. Frozen plasma samples were thawed at room temperature. The absorbance was determined against a blank containing 1000µl of FRAP reagent and 80µl of DDW. The sample preparation contained 20µl of the sample, 60 ul DDW, and 1ml of FRAP reagent. The standard curve was calibrated using a series of standards from diluting 1mM ferrous sulfate solution. The concentration for the series of dilutions included: 0.1, 0.2, 0.4 at increasing increments until the concentration reached 1.0mM. FRAP values were determined by UV-visible spectrophotometer (Thermo Scientific, BioMate 3S cat. # 840-208400) read at 593nm. An average FRAP value of the triplicate samples was determined for each individual measured. Each study group average FRAP values were determined and overall mean for each case group was calculated.

Hormonal Assays using ELISA kits

Leptin (Life Technologies, cat. # KRC2281), FSH (Biotang, cat. # R6403), LH (Shibayagi, cat. # AKRLH-010S) and estrogen (Calbiotech, cat. #ES180S-100) were measured using rats' ELISA kit according to manufacturers' protocols with microplate reader.

Tissues Harvesting and Analysis

Tissues removed from the rats such as ovary, hypothalamus were immediately snap frozen in liquid nitrogen and stored at -80 ^{o}C for homogenization and Western Blotting. The remaining body carcasses were stored at -80 ^{o}C for later proximal analysis

to determine the percentage of crude protein, fats, minerals, total calories and fatty acids profiles of isolated fat.

Western Blotting for Expressions of E-cadherin, β- catenin and Leptin Receptor

E-cadherin, β-catenin (from the ovary) and leptin receptors (from the hypothalamus) expressions were examined using Western blots. Each sample was homogenized using RIPA lysis buffer (Triton X-100 10%, Sodium Deoxycholate1%, SDS 0.10%, NaCl 0.15M, NaH2P04 0.01M, NaF 50mM, EDTA 2mM) containing protease inhibitor cocktail (Thermo scientific, cat. #78442) and the cell lysates extracted upon refrigerator centrifugation. The protein concentration of the cell lysates was determined by bicinchoninic acid (BCA) assay protein (Thermo scientific kit, 23236). Protein samples were run with ladder (Fisher scientific, cat # BP 3603-500) and separated by electrophoresis on 8-12% SDS-polyacrylamide gel and electro-transferred onto hybond-ECL nitrocellulose membranes. Blotted membranes were blocked for 1hr with 2% non-fat milk at room temperature. The membranes were washed in 10% TBST contains 1xTris buffered saline (Mediatech, Inc. cat. #46-012-CM) with 10%Tween® 20 (Acros, cat #9005-64-5) three times at 5mins each. They were incubated with primary antibodies diluted 1:1000 (5ug of primary antibody to 5ml TBST) for E-cadherin and β-catenin while leptin receptor at 1:2000 overnight at 4^{o}C. The primary antibodies used were mouse antibody (Cell Signaling Technology Cat#14472s) for E- cadherin, rabbit antibodies (Cell Signaling Technology Cat# 8480S RRID:AB_11127855) for β-catenin and leptin receptor (rabbit IG) (Thermo Fisher Scientific Cat# PA1-053 RRID:AB_2296991). β-actin (Sigma-Aldrich Cat# F3022 RRID:AB_476970). After primary antibody incubation, the blots were washed three

times in TBST for 5minutes each before been probed with secondary antibody conjugated with horseradish peroxidase at a dilution of 1:10000 (5ug to 50ml of TBST plus with 2% non-fat milk) at 4^0c overnight. The secondary antibodies used are goat anti-mouse (Cell Signaling Technology Cat# 7076S, RRID:AB_10695470) for E-cadherin and goat anti-rabbit (Thermo Fisher Scientific Cat# 31460 RRID:AB_228341) for β-catenin and leptin receptor. After the incubation with secondary antibody, it was washed three times in TBST at 5minutes each. The HRP-conjugated antibody–chemoluminescence reagent, Lumigen TMA-6 solution A and B (Catalog no TMA-100) from Lumigen® ECL Ultra was used for visual development by mixing 1 parts of solution A to 1 part of solution B. The substrate was incubated on the membranes for 5minutes under dark. Excess solution was removed and blot images were obtained using chemoluminescence digital imaging, Fluorchem M. Protein simple (Santa Clara, CA, U.S.A).

Total Body Assay

The total body and feeds compositions were conducted using proximal analysis techniques; Soxhlet method was used to evaluate percentage crude fat, Kjeldahl method for percentage of crude protein, bomb calorimetry for total calories and “ashing” for total inorganic content.

Sample Preparation for Proximal Analysis

Whole animal carcass and feeds were weighed and freeze dried using a freeze dryer (Labconco Freezone 2.5 Cat.# 7670520) (Fig. 6) to remove the water content. Whole animals’ carcasses and feeds were reweighed after drying. The samples were then grounded, sieved and then stored at -30 ^{0}C for later analysis.

Fig. 6. Samples on freeze dryer (Labconco Freezone 2.5)

Kjehldhal Analysis

This method is used to evaluate the percentage of crude protein present in the sample.1000-1200mg of the sample was measured into Kjeldahl flask (place flask in 250ml beaker to hold upright).1.9 g of K_2SO_4, 80 mg of mercuric oxide, 2 ml of concentrated H_2SO_4 and 2 boiling chips were added into the flask with gentle mixing for 1-2 minutes. The Kjeldahl flask was placed on the burner with the neck of the flask inserted into the glass manifold. The heater was switched to 3 with the air on under the hood. It was refluxed for 8 to 12 hours (sample will look clear with black specks). It was allowed to cool, 10ml of deionized distilled water was added and the mixture was filtered. It was distilled by placing beaker with 5 ml of boric acid with several drops of Kjeldahl indicator at the bottom of the apparatus. The sample was added slowly to the top of the distilled apparatus. 10ml of $NaOH/Na_2O_3S_2$ was added slowly (solution

turned black). The total volume of boric acid was allowed to measure 20-30ml, blue in color. The solution was titrated back with 0.1N dilute HCl until changed to steady pink. The samples were run in duplicates. Percentage of crude protein was calculated using the nitrogen content of the sample as follows;

Nitrogen (g)/kg = (ml-HCl – ml blank) x Normality x 14.01/ weight (g) x aliquot volume.

Soxhlet Analysis

This method is used to determine the percentage of fatty acids present in a sample. 2.5g of the sample was mixed thoroughly with 2.5g of anhydrous $NaSO_4$ in mortar and pestle. 2g of the mixture was placed into cellulose thimble, top with glass wool, and labeled the thimble with a pencil. 175ml of petroleum ether (PET) was measured into the round bottom flasks with 2 boiling chips and the thimble was put into Soxhlet sample extraction chamber. The assembled apparatus includes; round bottom flask (seated at the base), Soxhlet extraction chamber (middle), and condenser on top. Each condenser was attached firmly to each other by tubing. The water was turned on and a steady stream of water going through it with the light on. The mantle heater was turned to 3 and allowed to reflux for 8-12 hours. After reflux, it was allowed to cool off (10-20 minutes) by turning off the heat, drained the fluid from thimble into Soxhlet extraction chamber and finally into round bottom flasks. The drained thimbles were placed in an oven at 60 ^{0}c for 24 hours, allow to cool in a desiccator for about 1-2hours and then reweighed. The samples were run in duplicates. The percentage of crude fatty acids was calculated as;

Soxhlet = ((weight. before extraction (g) –Weight. after extraction (g))/ Weight before extraction) x 100

Ashing Analysis

This method is used to evaluate the percentage of crude organic minerals present in a sample. Crucible was first weighed. 5g of the sample was transferred into the crucible and then placed in an oven (Heat) at 700^0C for 5 – 24 hours. The crucible plus sample was placed in a desiccator (1-2 hours) after "ashing" to cool and then reweighed. The percentage organic minerals (Ash) is calculated as:

Percentage Ash = ((Weight before- Weight after ashing)/ weight before)) x100.

Bomb Calorimetry

This method is used to measure the total calories present in a sample. About 1 – 1.2 g of sample was measured into combustion capsule. The combustion capsule was then placed into ringer holder. The ignition thread was twisted around the wire and draped into combustion capsule, making sure it touched the sample. The valve was closed and charged with oxygen. 2000ml of deionized distilled water was added into the chamber (water) bucket. "bomb" was placed into water bucket and ignition wires were attached. The sample weight was input into the bomb calorimeter computer and start sequence (Fig. 7). The "bomb" explodes the sample and print off the results. The bomb was removed from the bucket and slowly release the combustion capsule assembly. The "bomb housing" and combustion capsule assembly was rinsed with deionized distilled water into a 250ml beaker. 2-4drops of methyl red indicator was added into the

beaker and then titrated against 0.1N of NaoH until a yellow uniform color change is observed. The samples were run in duplicates.

Total calories of dry matter = Calories on receipt – ml of titration (nitrogen)

Fig. 7. Paar 6200 Automatic Isoperibol Oxygen Bomb calorimeter.

Statistical Analysis

The Mean value and standard error of the mean were calculated for leptin, body weight, food intake, spleen weight, FRAP, organic minerals, FSH, LH, estrogen, total calories, crude protein, fatty acids, and CoQ_{10}. Data was analyzed using general descriptive models program in SPSS version 23 (IBM Corp. Armonk, NY, USA) and Graph Pad statistical software (Prism 6 for window version 6.07 Graph Pad, CA) using multiple comparisons (uncorrected Fisher's LSD). The level of significance was determined at $p<0.05$ for all analysis.

CHAPTER 3

RESULTS

Leptin Regulates Body Weight

The average body weight of all the groups were monitored weekly before and after the treatments with digital weighing scale. There was a decrease in the body weights of the groups treated with HFD leptin and HFD leptin+CoQ_{10} when compared with the control (HFD) at the 16th week when the treatments started (Fig. 8). This correspond to previous published work showing that leptin decreases the body weight of animals [25,31,58]. Therefore, leptin plays a significant role in the regulation of body weight. However, CoQ_{10} did not have an effect on body weight when used alone (Fig. 8).

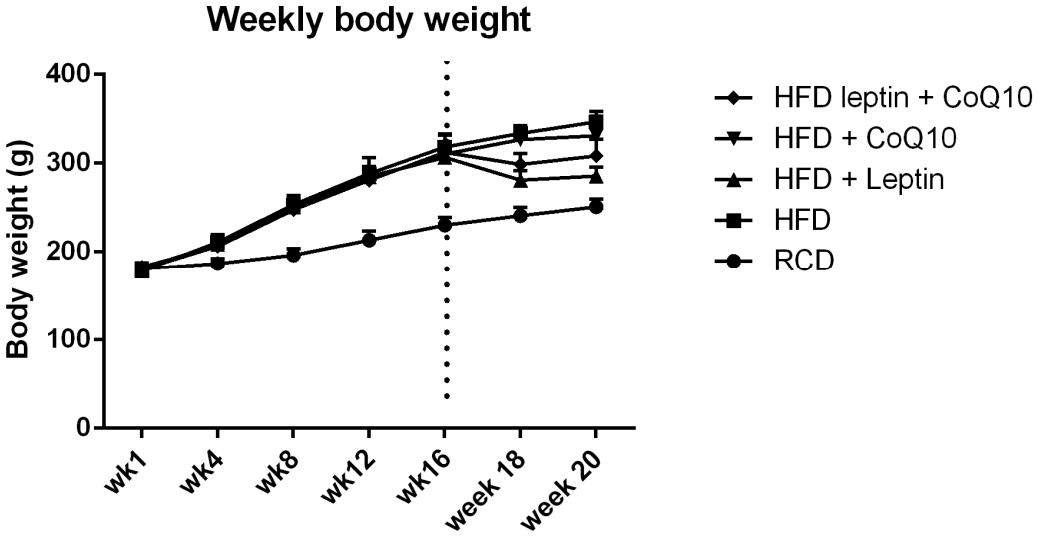

Fig. 8. Effects of leptin and CoQ10 on the body weight.

Table 2: Analysis of average weekly body weight of rats in grams and reported as the mean ± standard error of the mean.

GROUP	WEEK 1	WEEK 4	WEEK 8	WEEK 12	WEEK 16	WEEK 18	WEEK 20
RCD	180.03±3.55	186.04±2.97	195.98±3.74	212.70±5.44	229.73±4.46	240.52±4.86	250.34±4.61
HFD	177.91+1.61	210.38±3.07	252.71+3.69	287.94±6.33	317.82±5.16	333.17±3.17	346.18+4.14
HFD+LEP	181.16±0.94	208.03±5.25	250.6±4.13	284.33±4.18	306.01±5.99	280.40±5.47	285.26±4.97
HFDCoQ_{10}	179.93±1.61	206.24±5.59	248.11±6.45	281.71±7.00	309.93±6.00	326.16±7.78	330.53±7.51
HFDLEP+ CoQ_{10}	182.35±2.41	205.8±5.03	247.33±6.95	280.41±8.97	311.99±11.27	298.26±7.08	307.88±10.80

Leptin Regulates Food Intake

In this study, the average weekly food intake was measured using a digital weighing scale. It was observed that there was a decrease in the food intake among the treatments groups of HFD leptin and HFDleptin+CoQ_{10} when compared with the control, HFD at the 16th week when the treatment started. This correspond to previous published work[28] showing that leptin is involved in the decrease in the appetite of the rats which ultimately decreases their food intake, while CoQ_{10} did not show effects on food intake in all the groups when used alone (Fig. 9). It was also observed that the RCD control group consumed more food that the HFD groups, but this did not transform to increase in their body weight due to lower fats and calorie contents in their feed unlike the HFD (Fig. 9).

Table 3: Analysis of average weekly food intake of rats in grams and reported as the mean ± standard error of the mean.

Group	WK 1	WK 4	WK 8	WK 12	WK 16	WK 18	WK 20
RCD	17.52±1.69	15.65±0.68	15.95±0.63	16.37±1.28	16.57±0.68	16.15±0.58	15.87±0.73
HFD	17.76±1.16	10.46±0.34	10.68±0.25	10.47±0.33	10.41±.40	9.85±0.36	10.43±0.52
HFD+LEPT	16.85±1.35	11.7±1.2	11.6±1.80	11.65±0.05	11.2±0.4	7.5±0.3	8.9±0.3
HFD+CoQ_{10}	16.67±0.83	10.32±0.20	10.3±0.11	10.85±0.69	10.025±0.46	9.5±0.28	9.76±0.16
HFDLEP+ CoQ_{10}	15.4±1.57	10.76±0.17	11.53±0.27	11.33±0.63	11.40±0.15	9.23±0.21	9.46±0.46

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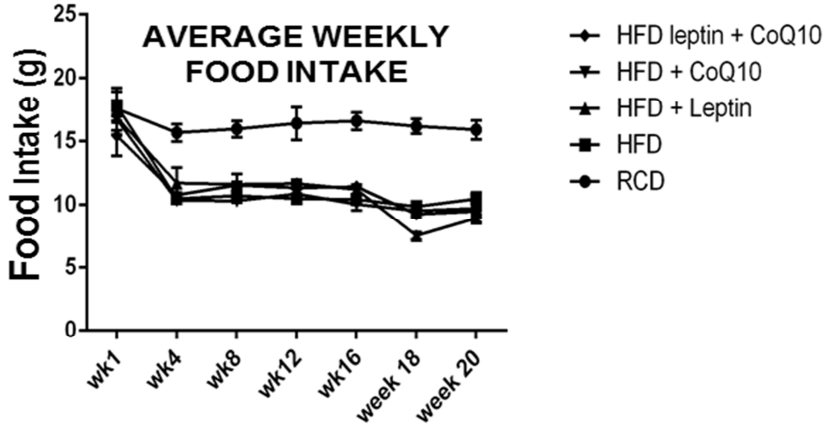

Fig. 9 Effects of leptin and CoQ_{10} on food intake.

There was a decrease in the food intake observed in the HFD leptin and HFD leptin+CoQ_{10} groups at week 16 of the treatment. This suggests that leptin plays a significant role in the regulation of food intake.

CoQ_{10} Increases Concentration of Plasma Leptin

The plasma leptin concentration was measured using ELISA. There was statistical significance increase *(p<0.05*) in the level of plasma leptin in the HFDCoQ_{10}+leptin treatment group *(p=0.03)* when compared with control HFD (Fig. 10). This suggests that CoQ_{10} enhances the concentration of leptin in the blood. Therefore, combination of leptin and CoQ_{10} increases the concentration of leptin in the blood.

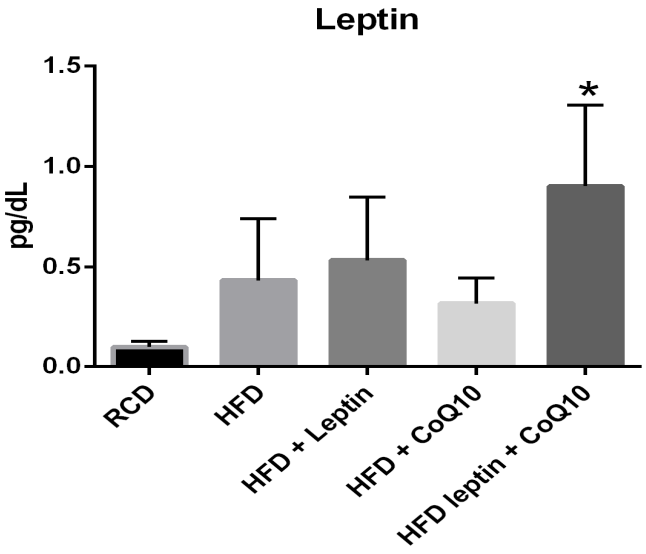

Fig. 10. Leptin concentration in the blood by ELISA.

The result showed an increase in the level of plasma leptin in HFDleptin+ CoQ_{10} treated groups when compared with HFD control group.

Reproductive Hormones Concentration in the Blood

Combination of Leptin with CoQ_{10} Regulates Follicle Stimulating Hormone (FSH)

Plasma FSH concentration was measured using ELISA. There was a significant increase *(P=0.0004)* in the level of plasma concentration in the HFD leptin+CoQ_{10} group

when compared with the control HFD (Fig.11). This suggests that combination of leptin and CoQ_{10} could enhance the concentration of FSH in the blood.

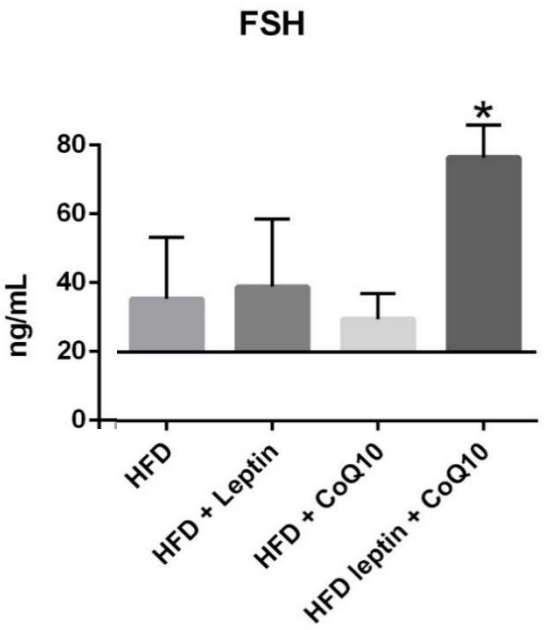

Fig. 11. Follicle stimulating hormone (FSH) concentration by ELISA.

The results showed an increase in the level of FSH in HFD leptin+ CoQ_{10} treated group *(P=0.0004)* when compared with HFD control group.

Regulation of Luteinizing Hormone (LH) by Leptin with CoQ_{10}

Plasma LH was measured using ELISA. Leptin plus CoQ_{10} treated group showed an insignificant increase *(p> 0.05)* in the level of plasma LH when compared with the control HFD (Fig. 12). This suggests that leptin+CoQ_{10} may not have effect in the regulation of LH in the blood.

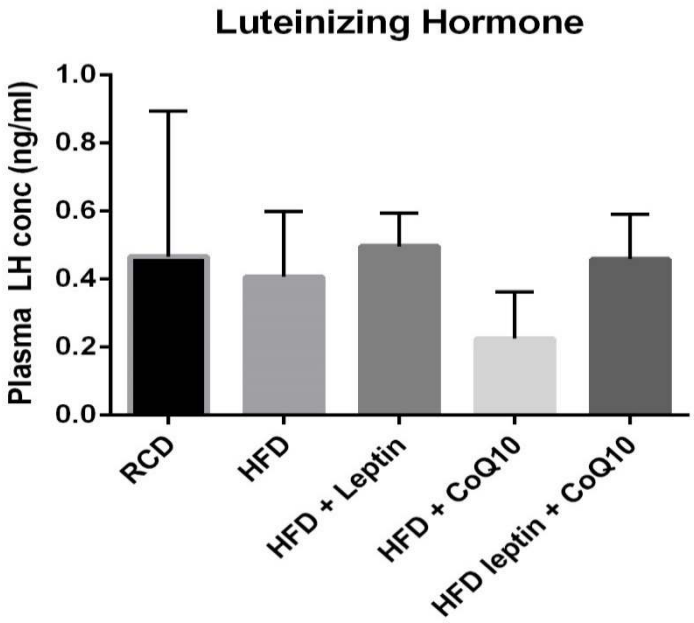

Fig. 12. Plasma luteinizing hormone (LH) concentration by ELISA.

The results showed statistically insignificance (*p>0.05)* increase in the level of LH in HFD+ Leptin-treated group followed by HFD leptin+ CoQ_{10} when compared with HFD control group.

Regulation of Plasma Estrogen Hormone (EH) By Leptin with CoQ_{10}

Plasma EH was measured using ELISA. There was a slight insignificant increase *(p>0.05)* in the level of EH in HFD leptin+CoQ_{10}, HFDCoQ_{10}, and HFD leptin treated group when compared with the control HFD. This suggests that Leptin plus CoQ_{10} may not have an effect on the regulation of plasma EH (Fig. 13).

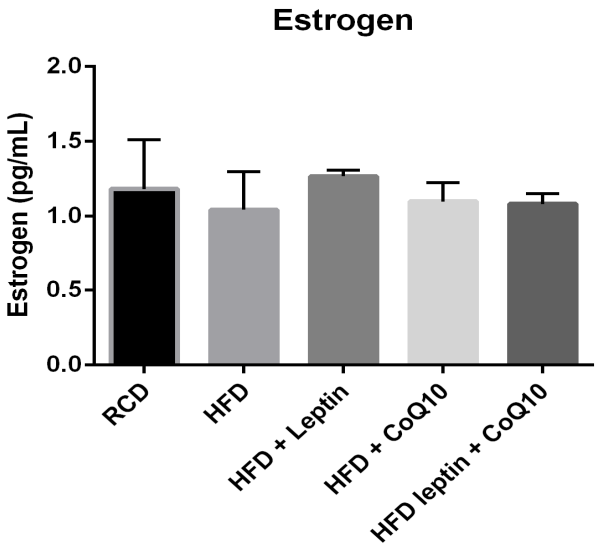

Fig. 13. Plasma estrogen concentration by ELISA.

The result showed an insignificance increase *(p>0.05)* in plasma estrogen in the leptin-treated group when compared with HFD. However, no statistical significance was observed *(p>0.05)* among the treated groups when compared with the HFD control group.

Total Body Composition of Rats using Proximal Analysis

This is a system for approximating the value or materials of the body (animals, foods) based on the body components. For this study, the rats' total body compositions were analyzed into different fractions according to their feeding values. The fractions analyzed include; crude protein, crude fats (ether extract), inorganic content (ash) and total calories.

Effect of Leptin with CoQ_{10} on Crude Fat

The rat's total body crude fat was estimated using the Soxhlet method. There was significance increase, *p=0.01* in the percentage of crude fats in the HFDleptin+CoQ_{10} treated group when compared with HFD control group (Fig. 14). The result suggests that leptin+CoQ_{10} could increase the percentage of total body crude fat.

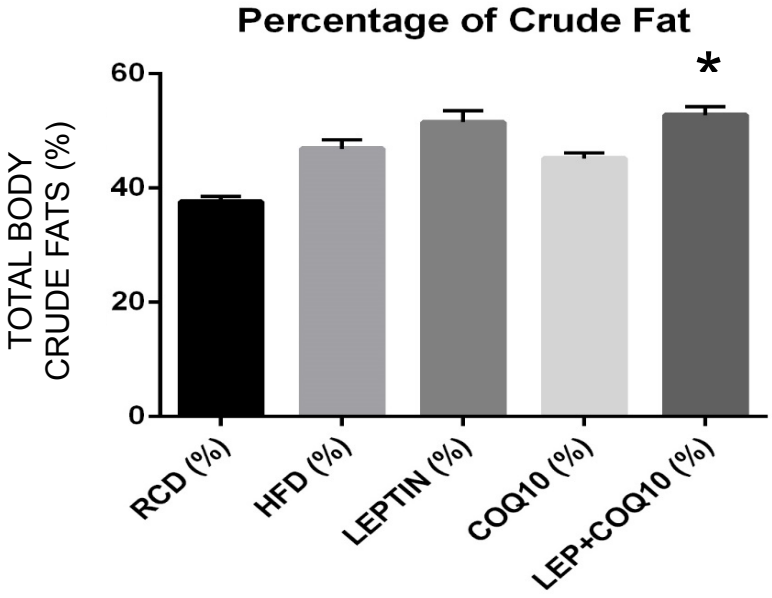

Fig. 14. Total body crude fat measurement by Soxhlet method.

The result showed statistical significance increase *(p<0.05)* in HFD leptin+ CoQ_{10} *(p=0.01)* treated group when compared with HFD control group.

Effect of Leptin with CoQ_{10} on Crude Protein

The percentage crude protein was assayed using Kjeldahl method. The result showed no significance difference in the treated HFD leptin+CoQ_{10} when compared with the control HFD (Fig. 15) *(p>0.005).* Hence, leptin combined with CoQ_{10} does not

regulate crude protein in the body because CoQ_{10} functions as an antioxidant and electron transport chain enhancer.

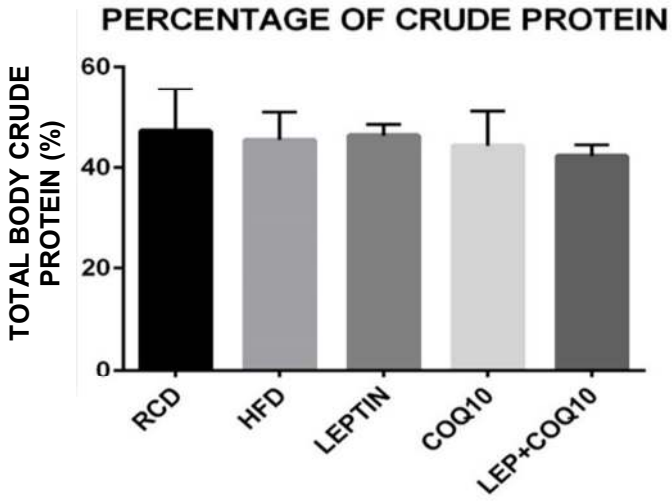

Fig. 15. Total body crude protein by Kjeldahl method

The result showed no statistical significance, *p>0.05* in all the treated groups when compared with HFD control group.

Effect of Leptin and CoQ_{10} on Total Body Inorganic Content.

The percentage of total body minerals was estimated using “ashing” method. The result showed no statistical significance, *p>0.05* in the treated group HFDleptin+CoQ_{10} when compared with HFD control group (Fig. 16). This shows that combination of leptin and CoQ_{10} does not have an effect on the inorganic content of the body. However, the higher amount seen in the RCD when compared with HFD may be connected with HFD tying up minerals (fat) in the small intestine and excreting them.

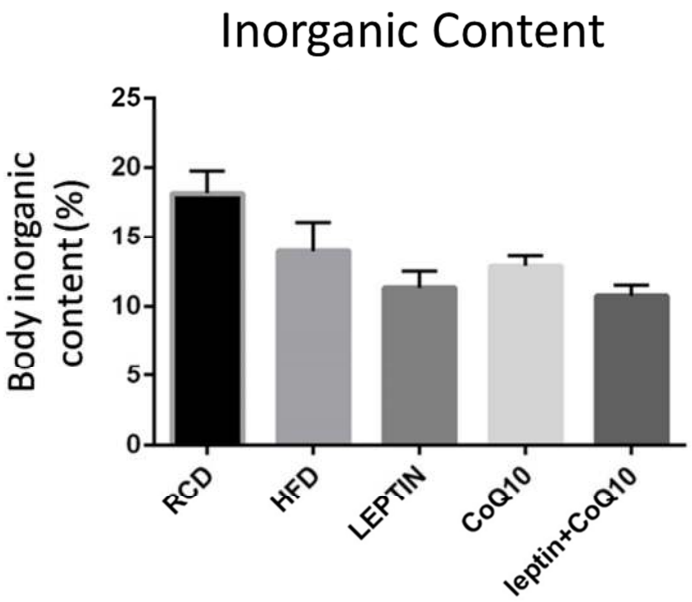

Fig. 16. Estimation of body inorganic content using "ashing" method.

The result showed no statistical significance, *p>0.05* in all the treated group when compared with HFD control group.

Leptin Combined with CoQ_{10} Regulates Total Body Calories

The total body calories were estimated using the "bomb" calorimetry method. The result showed a significance increase in the total calories of the HFDLeptin+CoQ_{10} (p=0.0006) treated group when compared with the HFD control group (Fig. 17). This indicates that combined leptin and CoQ_{10} could increase in total body calories.

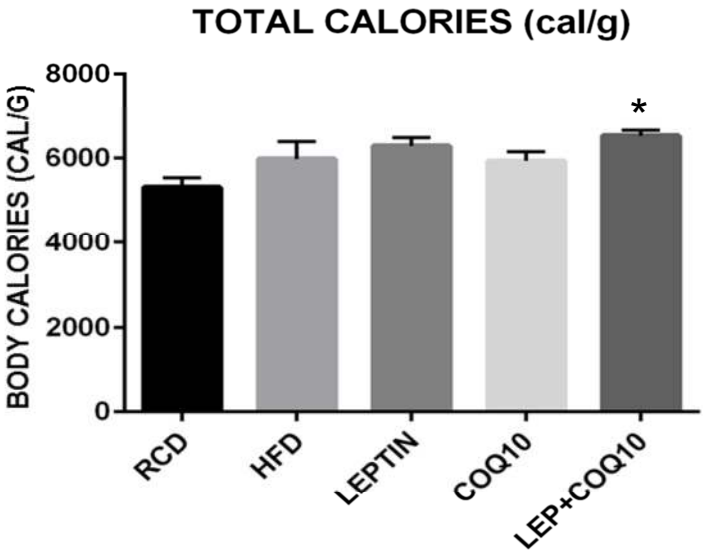

Fig.17A. Total body calories estimation using the "bomb" calorimetry method.

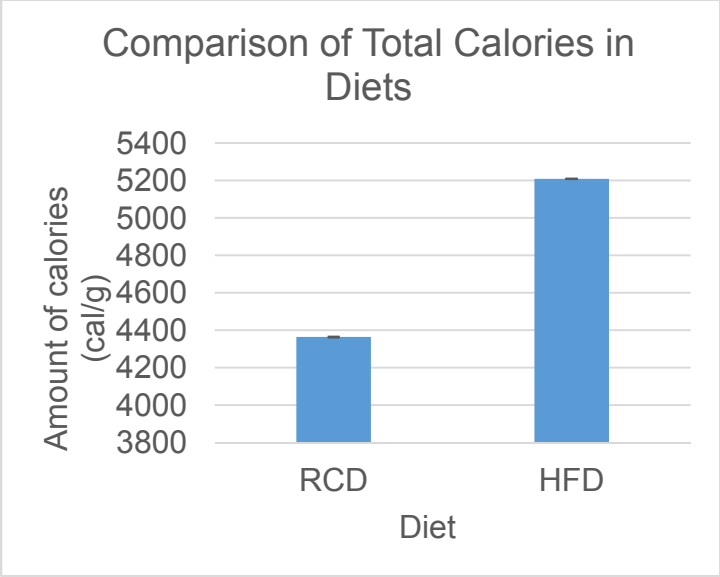

Fig.17b. Comparison of average total calories in diets (RCD and HFD) consumed by the rats. The HFD has more calories than RCD.

Table 4: Comparison of average total calories in diets (cal/g) between regular chow diet (RCD) and high-fat diet (HFD), reported as the mean± standard error of the mean.

RCD	HFD
4363.75±2.83	5209.335±2.21

CoQ_{10} is involved in immunological reaction of the body

The weight of the spleen was measured using a digital weighing scale. The result showed that there was a significant weight increase ($p<0.05$) in the spleen weight of $HFDCoQ_{10}$ *($p=0.0016$)* and $HFDleptin+CoQ_{10}$ (*$p= 0.002$)* treated groups when compared with the HFD control group (Fig 18). This suggests that CoQ_{10} has an effect on immunological response in the body and thus improved immune function in the rats. The effect may be attributed to splenomegaly observed in $HFDCoQ_{10}$ and $HFDleptin+CoQ_{10}$ treated groups directly or partially caused by CoQ_{10} action in the body.

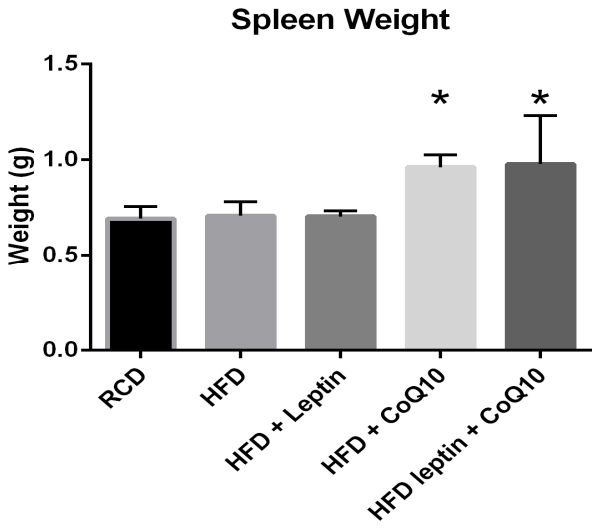

Fig. 18. Graph showing spleen weight measured by digital weighing balance

The results showed statistical significance increase in the spleen weight in HFDCoQ_{10} *(p=0.0016)* and HFDleptin+CoQ_{10} *(p= 0.002)* groups when compared with HFD control group.

Table 5: Analysis of average spleen weight in grams and reported as the mean± standard error of the mean.

RCD	HFD	HFD+LEP	HFD+COQ10	LEP+COQ10
0.69225±0.031	0.706375±0.026	0.7035±0.020	0.961±.032	0.9767±0.145

Measurement of Total Antioxidants

Ferric reducing ability of plasma (FRAP) assay was used to estimate the total antioxidant present in the plasma. The ferric reducing ability of plasma in the HFDleptin+CoQ_{10} treatment group showed a statistical significance increase ($p<0.05$) when compared with the HFD control group (Fig. 19). This result suggests that combination of leptin with CoQ_{10} could help to enhance the protective effect of CoQ_{10} against free radicals that causes DNA damage in tissues.

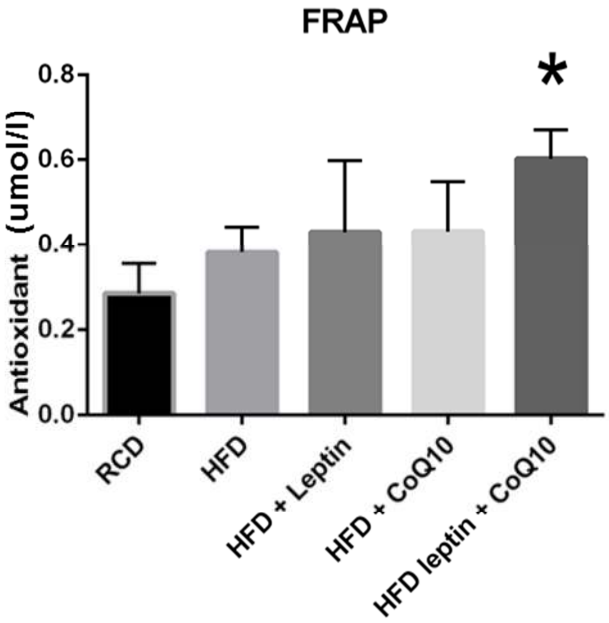

Fig.19. Plasma antioxidant concentration measured by FRAP assay.

The result showed statistical significance increase (*p=0.0016)* in the level of antioxidants in HFD leptin+ CoQ_{10} treatment group when compared with HFD control group.

Expression of Beta Catenin and Epithelium Cadherin in the Rats' Ovary

In the study, the expression of β- catenin and E-cadherin were analyzed by Western blot using the rats' ovary lysate.

CoQ_{10} Down-Regulate the Expression of Beta Catenin in the Ovary

The result showed that CoQ_{10} down-regulated the expression of β- catenin in the ovary (Fig. 20). β-catenin expression was higher in rats fed with HFD. However, there was a decrease in expression in the HFDleptin+CoQ_{10} treatment group, followed by

HFDCoQ$_{10}$ and then HFD leptin treatment group when compared with HFD group (Fig. 20). This shows that CoQ$_{10}$ down-regulates the expression of β-catenin in the ovary.

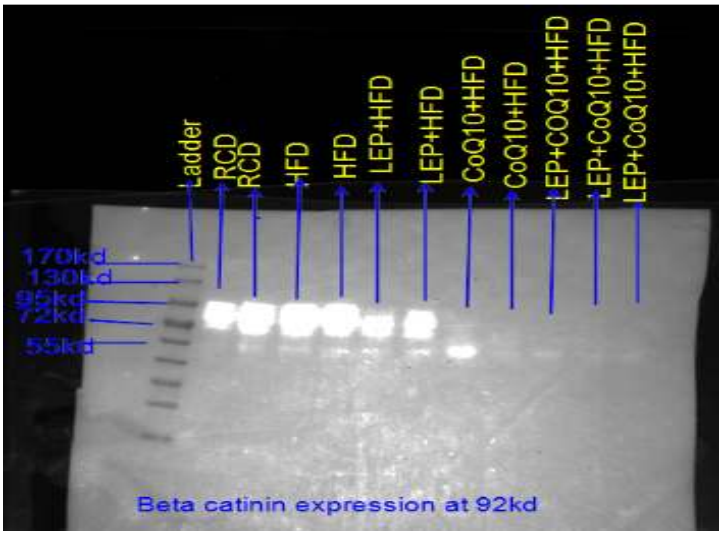

Fig. 20. Expression of β- catenin in the ovary by Western blot

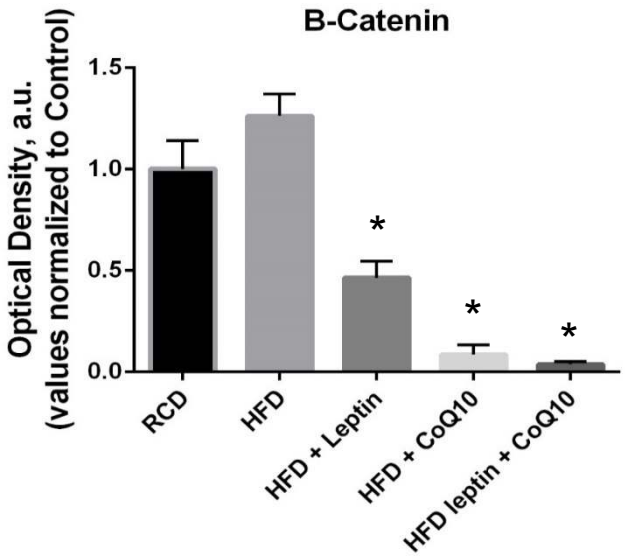

Fig. 21. β- catenin quantification in the ovary

Quantification of the β-catenin showed significance decrease *(p<0.05)* in the expression of β- catenin in leptin HFD, CoQ$_{10}$ HFD and leptin+CoQ$_{10}$+HFD treated

groups when compared with HFD control. This shows that CoQ_{10} downregulates the expression of β- catenin in the ovary.

Expression of E- cadherin in the ovary

In this study, expression of E-cadherin in the ovary was examined using western blot. The result showed no detection of E-cadherin expression in all the groups at 135kd (which is the size at which E- cadherin was to be expressed according to the protocol used). However, multiple repeated analysis of the lysate for E-cadherin showed consistent detection of an unidentified protein band expressed with E-cadherin antibody at 50kd in all the groups (Fig. 22).

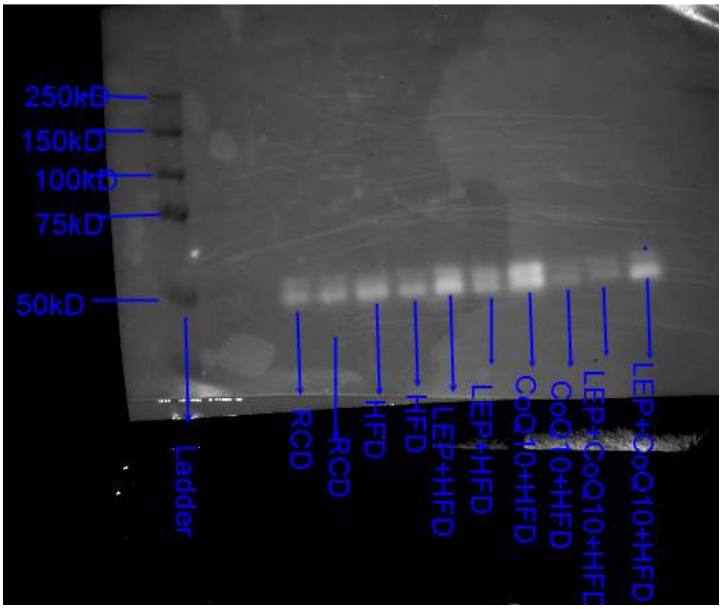

Fig. 22: Western blot showing band of unknown protein at 50kDa that was expressed with E-cadherin antibody.

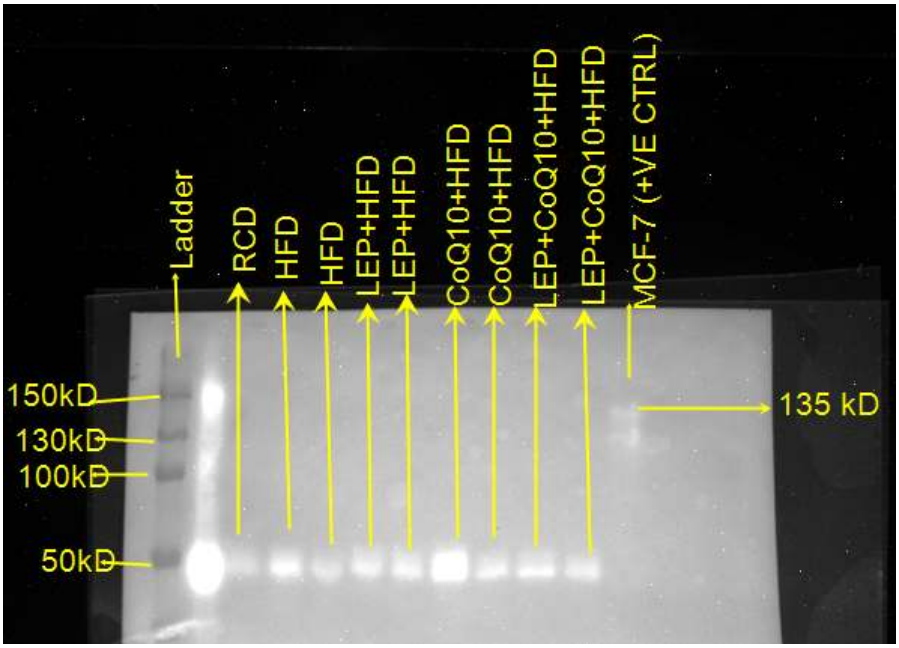

Fig. 23. Western blot showing expression of E- cadherin with positive control (MCF-7)

A positive control, MCF-7 was run along with the samples. The positive control showed expression of E-cadherin at 135kDa while samples showed expression of an unknown protein that binds with E-cadherin antibody at 50kDa.

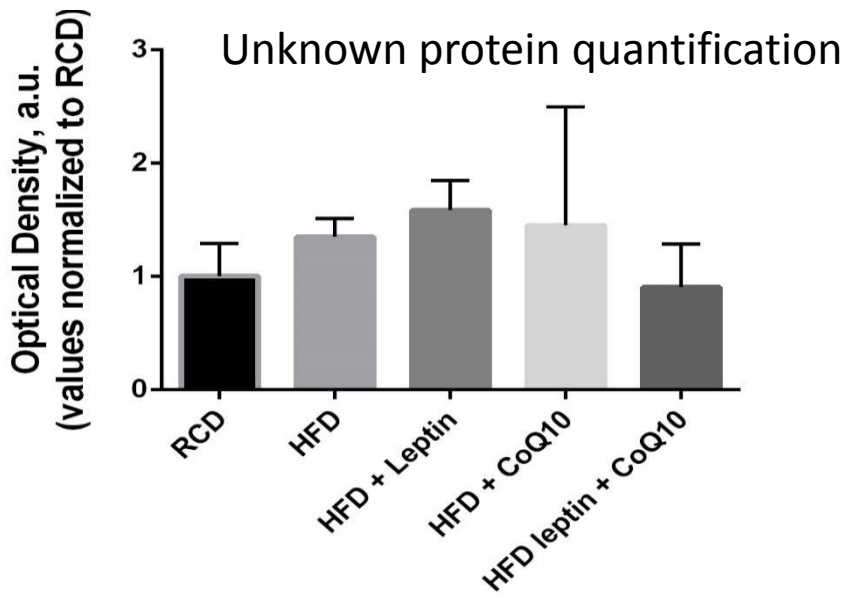

Fig. 24: The unknown protein quantification in the ovary that shows expression with E-cadherin antibody.

Quantification of the unknown band showed no significance *(p>0.05)* among the groups. However, there was a decrease in the expression of the band in HFDleptin+CoQ_{10} and slight insignificance increase *(p>0.05)* in the expression between HFD Leptin and HFD+CoQ_{10} treated groups when compared with HFD control group (Fig. 24). This suggests that CoQ_{10} might decrease the expression of the band when combined with leptin.

Leptin Receptor (LEPR) Expression in the Hypothalamus

Hypothalamus lysate was analyzed for expression of leptin receptor (LEPTR). In this study, expression of long isoform (125KDa) and a short isoform (100kDa) of LEPTR was checked. The LEPTRs, long and short forms were not expressed at the sizes; 125kDa and 100kDa respectively. However, there were small unknown proteins that showed expression with leptin receptor antibody detected at sizes smaller than 70kDa.

(Fig. 25). The protocol for the assay indicated that two smaller unrelated proteins could be detected at 31kDa and 35kDa. Quantification of protein detected at 50kD was done and analyzed (because of higher expression). The result showed downward regulation of the unknown LEPTR in HFDLeptin+CoQ_{10} and HFD leptin groups when compared with the HFD (Fig. 25).

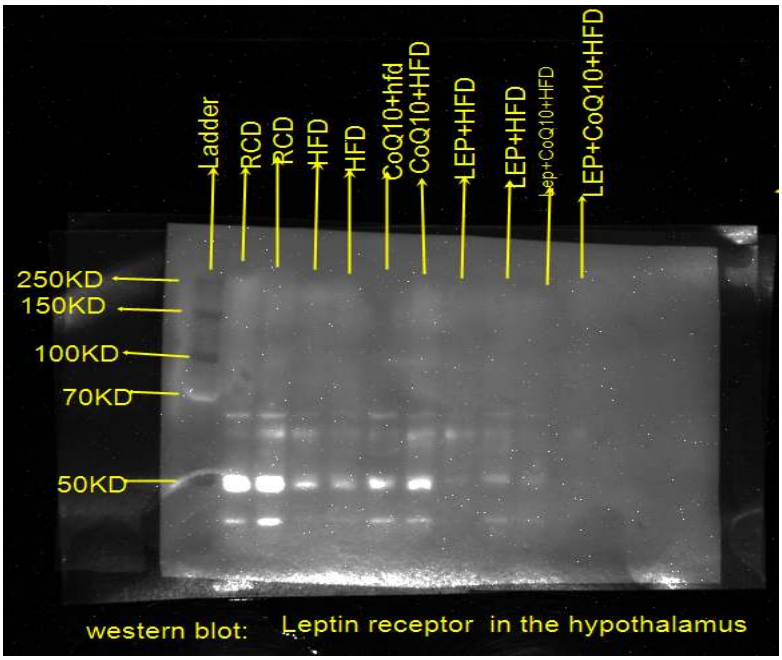

Fig. 25: Western blot showing expression of small proteins that binds with LEPTR antibody in the hypothalamus.

There was statistical significance *($p<0.05$)* in the upward regulation of the small LEPTR in RCD when compared with HFD group (Fig. 26). This shows that leptin tends to decrease the expression of this small protein detected at the 50kDa. There was a slight insignificant increase *($p>0.05$)* in the expression of HFDCoQ_{10} treatment group, when compared with the HFD group (Fig. 26).

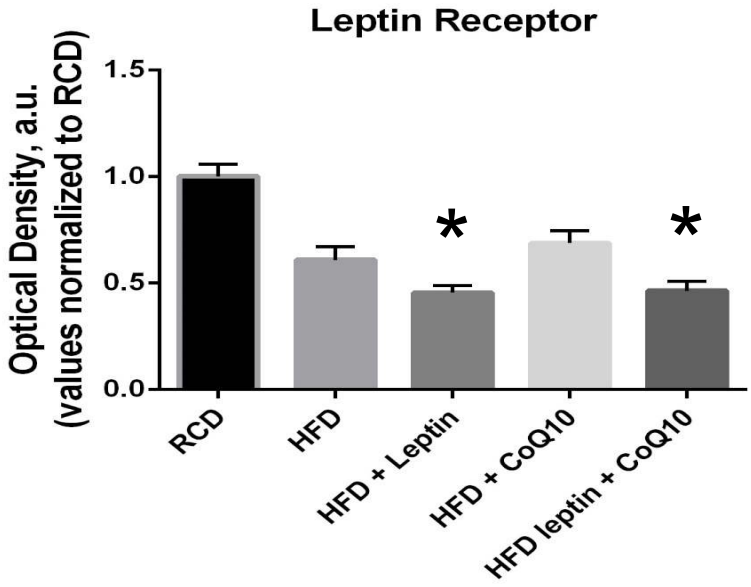

Fig. 26. Leptin receptor quantification in the hypothalamus

Quantification of the LEPTR showed significance decrease *(P<0.05)* in the expression of the small unknown protein in HFD leptin and HFD leptin+CoQ_{10} treated groups when compared with HFD control. From the graph above, it can be suggested that there may be less small isoform of LEPTRs (if identified as LEPTRs protein) that bind with the LEPTR antibody in the HFD leptin and HFD leptin+CoQ_{10} groups unlike the HFD group. The decrease in expression can cause delay in relay of information of energy status of the body to the brain, and this may have negative effect on hypothalamic-pituitary-gonadal axis of the body.

CHAPTER 4

DISCUSSION AND CONCLUSION

Leptin is a hormone synthesized primarily by adipose tissues and secreted into the blood stream to drive physiological functions of the body. Leptin helps to regulate food intake, body weight, immunological responses as well as infertility [6,36]. Leptin has been shown to reverse infertility as well as improve reproduction [82]. Coenzyme Q_{10} (CoQ_{10}) is an important component of electron transport chain in the mitochondria of human and animal body cell. CoQ_{10} helps in the synthesis of ATP and acts as a powerful antioxidant and quenches free radicals in the mitochondrial and lipid membranes [62]. Research has shown that CoQ_{10} helps in reproduction as well as improved fertility [62]. It has been suggested that antioxidant such as CoQ_{10} supplementation could alternate infertility and improve reproduction [62,69]. Therefore, this study focused on the combination effects of CoQ_{10} and leptin on infertile female rats. Various results from this study indicate that combination of leptin with CoQ_{10} can play a significant role in biological functions that could results in an increase in reproductive functions as well as fertility.

The first observation from the study was the role of leptin in the regulation of food intake, body weights, and energy expenditure. The food intake and body weight of the rats were monitored before and after treatment. This was measured to determine if leptin, CoQ_{10} and leptin plus CoQ_{10} reduce food intake and body weight. Previous researches have shown that leptin plays a key role in regulatory centers to reduce feed intake, regulate the body weight and energy expenditure of animals [25,31,58]. Results (Fig.

VIII) showed that reduction in the body weight started at week 16 when the treatment started among the treated groups, HFD leptin (280.41 ± 5.47), and HFD leptin+CoQ_{10} (298.26±7.08) but not in the HFD+CoQ10 (326.16±7.78) treated group and the control group, HFD (333.17±3.17) (Table 2). It was also observed that decrease in feed intake (Fig. IX) started at week 16 when the treatment started among the treatment groups; HFD + leptin (7.5±0.3) and HFD leptin+CoQ10(9.23± 0.21) but not in the HFD+CoQ10 group (9.5±0.28) and the control group, HFD (9.85±0.36) (Table 3). This result is in consistent with the study conducted by Pelleymounter et al. (1995)[58] and Halaas et al. (1995) [31] that leptin regulates body weight and food intake animals. Coll, Farooqi, and O'Rahilly (2007)[19] findings also showed that leptin plays a major role in the downstream anorexigenic pathways in the reduction of food intake in animals. From this study, CoQ_{10} does not have an effect on the body weight and food intake when administered alone. This result was in line with review by Abdali, Samson, and Grover (2015) [1] that CoQ_{10} has no effect on the body weight and fat mass. In recent research conducted by Gopi, Purushotha, and Chandrasek (2015)[8] in broiler chicks, it was observed that CoQ_{10} does not have an effect on the feed intake or body weight when administered alone.

The role of leptin in fertility was also observed in this study. Previous researches have shown that leptin administration reverses obesity-induced infertility [25.35,82]. The irregularity of the oestrus cycle was first noticed at the end of the 12th week in the HFD groups. At the 16th week, it was observed that the rats in the HFD groups had disrupted estrous cycle indicating infertility. Normal regular oestrus cycle in female rats is between 4-5 days. Oestrus cycle consists of proestrus, estrus, metestrus and diestrus phases. The continuation of a particular phase of the cycle for more than 2 days or skipping of

estrus phase signified infertility in the rats. Leptin administration restored fertility and oestrus cycle became regular in the HFD leptin and HFDLeptin+CoQ_{10} treatment groups. It was observed that the HFD control group still maintained the irregular oestrus cycle 2 weeks after treatment. This result supported the study conducted by Chehab (1997)[18] in obese mice and Hoggard, Hunter, Trayhurn, Williams, and Mercer (1998)[35] that administration of exogenous leptin reverses obese-induced infertility in animals.

Previous researches have demonstrated that circulating leptin level in the blood is lower in males and post-menopausal women when compared with females and premenopausal women respectively [52,54]. In addition, when leptin signaling is disrupted in rats they do not experience puberty. This is an indication that leptin regulates gonadal function and reproduction, its deficiency or resistance can result to reproduction abnormalities [52,54]. Studies have shown that leptin has an effect on gonadotropin-releasing hormone (GnRH) at the anterior pituitary gland, thus stimulating the secretion of serum FSH, LH [54] and estrogen [65]. CoQ_{10} can also help in reproduction [69]. CoQ_{10} helps in synthesis of ATP needed by tissues (oocyte) for the process of ovulation, implantation and embryo development [10]. In this study, it was observed that there was an insignificant increase *($p>0.05$)* in the level of plasma LH in the HFD leptin and HFD leptin+CoQ_{10} treatment groups when compared with the control HFD. This result showed that leptin may induce plasma LH concentration. The insignificant increase observed might be connected to the follicular stage when the sample was collected because LH and FSH tend to increase from early follicular stage – mid follicular-ovulation and the peak observe at the luteal stage of follicular developement before declining back to the early follicular stage [29]. Results from Moschos, Chan, and

Mantzoros, (2002)[54]; Yu, Kimura, Walczewska, Karanth, and McCann, (1997)[80]; Donato et al. (2009)[22] studies indicated that leptin stimulates the production of LH and FSH in the blood. The result (Fig.11) from plasma FSH concentration showed significance increase *(p<0.05)* in plasma FSH in HFDleptin+CoQ_{10} treated group when compared with the HFD control group. This indicates that combination of leptin and CoQ_{10} could stimulate an increase in the concentration of FSH in the blood. This is a novel finding as previous studies have suggested that CoQ_{10} if combined with another compound could help improve fertility [62,69].

While there are conflicting reports on the role of leptin on plasma estrogen, some researchers asserted that leptin does not have effects on plasma estrogen while other believe that leptin plays a role in the regulation of plasma estrogen[29]. The result from Shimizu et al. (1997) shows that leptin increases the level of estrogen in female rats [65]. The result of our study showed that leptin has a slight insignificance increase *(p>0.05)* in the level of plasma estrogen in the HFD leptin treatment group when compared with the control group, HFD. There was no significance difference *(p>0.05*) in the level of HFDleptin+CoQ_{10} and CoQ_{10} treatment groups when compared with the control, HFD. This suggests that leptin and CoQ_{10} may not have direct effects on plasma estrogen. However, this insignificant difference maybe dependent on the estrous stage which the sample was collected. Estrogen (like leptin) decreases feed intake, reduce body weight as well as energy expenditure [84]. Estrogen tends to induce leptin. The effects of estrogen can be mimicked by leptin action which can be the result of insignificant difference observed in estrogen level in HFD leptin and HFDleptin+CoQ_{10} when compared with HFD.

The observational changes noticed in the reproductive hormones from this study may be connected to the phase of the estrous cycle when the blood sample was collected. Leptin which is also a regulator of gonadal function and reproduction is dependent on the functional stage of the estrous cycle. Several studies have shown that leptin concentration increases from early follicular stage to mid-follicular to ovulatory and reach the peak level in early luteal phase and start declining in late luteal phase to early follicular stage, and this can be attributed with adipocyte leptin gene expression [44,52].

CoQ_{10} significantly increases *($p<0.05$)* the concentration of plasma leptin (Fig 10). This shows that combination of CoQ_{10} with leptin could enhance the effect of leptin in decreasing body weight, food intake, increasing energy expenditure as well as improving reproduction [69].

It was observed that combination of leptin with CoQ_{10} significantly increased *($p<0.05$)* the level of antioxidants present in the blood when compared HFDleptin+CoQ_{10} group with the HFD control group (Fig.19). This indicates that the combined effect of CoQ_{10} and leptin could help to reduce oxidative stress, the amount of oxidants and thus quench the free radicals that cause DNA damage especially in the reproductive organs and conversely promoting fertility. This is suggestive that higher amount of plasma antioxidant level could produce a positive effect in fertility and improve reproductive functions especially against oxidative stress and DNA damage in the reproductive system. This result is consistent with Tarnopolsky (2008)[69] submission that supplementation of antioxidants with another compound produces a more positive effect than lone administration.

Another observation from this study is that combination of leptin and CoQ_{10} significantly increases *(p<0.05)* the total body calories of the rats when compared with the control group. This may be due in parts to the higher level of body crude fat measured by Soxhlet (Fig. 14) as amount of calories tends to increase with an increase in fat.

From the study, it was observed that CoQ_{10} significantly increased *(p<0.05*) the spleen weight in HFDCoQ_{10} and HFDleptin+CoQ_{10} treatments group when compared with the HFD group (Fig. 18). This shows that CoQ_{10} increases spleen weight and therefore is involved in the immunological response of the body. This supports the previous observation by Gopi, Purushotha, and Chandrasek, (2015)[28] in broiler chick that CoQ_{10} supplementation at 20 mg kg^{-1} increases the spleen weight.

As expected, combination of leptin with CoQ_{10} does not have an effect on the total crude protein of the rats. There was no significance difference among the groups *(p>0.05)* (Fig. 15).

β- catenin and E-cadherin are among the major structural proteins found in the ovary to maintain epithelial integrity. They assemble to form cell to cell adherence junction for E-cadherin/ β- catenin complex in the ovary. E-cadherin/ β- catenin complex has been found to be involved in follicular development, ovulation and corpus luteum formation in immature rat ovary [67]. These proteins are also highly expressed in benign and malignant tumor epithelial cells [67], acting as a tumor suppressor[20].

One of the novel findings of the study is that CoQ_{10} down-regulates the expression of β- catenin in the rat ovary. It was observed that CoQ_{10} significantly down-

regulates ($p<0.05$) the expression of β- catenin in the treatment groups, HFDCoQ_{10} and HFD leptin +CoQ_{10} when compared with the HFD control group. Leptin effects also significantly down-regulates *($p<0.05$)* the expression of β- catenin in HFD leptin group when compared with the HFD control group. The observation from this study suggests that CoQ_{10} may have a negative effect on β-catenin in providing cell-to cell adhesion, ovulation as well as follicular development of the ovary. On the other hand, β-catenin (as well as E-cadherin) is mostly expressed (biomarkers) in metastatic tissues and cancer cells[67], the down-regulation of this protein maybe an indication for protective role of CoQ_{10} in the ovary.

The expression of E-cadherin was not detected at 135kDa, however, there was a consistent expression of an unknown protein at 50kDa which binds with E-cadherin antibody. The observation from this study suggests that E-cadherin may have a particular stage at which it is adequately expressed in the ovary since the follicular stage of development for the study was not established when the sample was collected. Research conducted by Davies (1998) compared the expression of E- cadherin in normal ovarian epithelium, benign and malignant ovarian tumor [67]. The result showed no expression of E- cadherin detection in four out of six normal ovaries examined and the other two ovaries showed very superficial expression. Although, E- cadherin expression was detected in normal fallopian tube and mammary gland (but not the ovary). However, expression of E-cadherin was detected in twenty-eight out of the thirty differentiated ovarian carcinomas examined [20]. In another study conducted by Sundfeldt, Piontkewitz, Billig, and Hedin (2000)[67] using rat ovary, E-cadherin expression was not detected in antral follicular but in the theca, interstitial cells, surface epithelium

of immature ovary and higher expression in benign and cancer cells [67]. E-cadherin and β- catenin serve as biomarkers for cancer and metastatic cells. Observation from this study suggests that E-cadherin may not be detected in normal rat ovary and also dependent on the follicular stage of development as well as the age of the ovary.

The expression of long isoform (125kDa) and short isoform (100kDa) leptin receptor (LEPTR) from the hypothalamus lysate was not detected in this study. However, small proteins (lesser than 70kDa) that bind with the polyclonal leptin receptor antibody were detected. These proteins may be small short isoforms of leptin receptors (*ob-Ra, ob -Rc, ob-Rd, ob-Re* or *ob-Rf*). The small protein which showed highest expression was quantified. The result showed statistical significance *($p<0.05$)* decrease in HFD leptin and HFD leptin+CoQ_{10} when compared with HFD control group. However, comparing the two control groups, the normal diet (RCD) showed statistical significance increase *($p<0.05$)* when compared with high-fat diet group (HFD). This result indicates that leptin may downregulate the small protein that binds with LEPTR antibody at 50kDa.

Conclusion

Overall, this research supports that combination of leptin with CoQ_{10} can play a significant role in biological functions that could results in an increase in reproductive functions as well as fertility in obese infertile female rats. The regular estrous cycle was restored after administration with leptin+CoQ10. The administration of combined leptin and CoQ_{10} increases the level of reproductive hormones, with significance increase *($p < 0.05$) i*n the level of follicle stimulating hormone while luteinizing hormone and estrogen

showed insignificance increase *(P > 0.05)* in the blood. These reproductive hormones help the ovary in folliculization as well as preparedness for ovulation. Leptin and CoQ_{10} increased the concentration of antioxidant and improved immune function in the rats.

Future Direction

This experiment should be carried out in a larger sample size with male /pregnant female models, evaluating the reproductive hormones and number of pups. Emphasis should be made on estrous cycle stages during collection of samples. The unidentified protein expression detected at 50kDa with E-cadherin antibody and the small proteins expressed below 70kDa with leptin receptors antibody should be analyzed for further studies.

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